Selection and Optimization of Hydrolysis Conditions for the Quantification of Urinary Metabolites of MDMA

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Abstract

Recovery of 3,4-methylenedioxyamphetamine (MDMA) urinary metabolites requires optimization of the hydrolysis of 4-hydroxy-3-methoxyamphetamine (HMA), 4-hydroxy-3-methoxymethamphetamine (HMAA), and 3,4-methylenedioxymethamphetamine (MDMA) conjugates prior to chromatographic analysis. Acidic and enzymatic hydrolysis with β-glucuronidase from Escherichia coli and Helix pomatia were evaluated. Acid hydrolysis yielded 40.0% and 39.3% higher HMA recovery compared to E. coli and H. pomatia hydrolysis, respectively (SE = 9.8 and 11.4%). E. coli β-glucuronidase hydrolysis MDMA recovery was 17.1% and 26.5% greater than acid hydrolysis and H. pomatia β-glucuronidase recovery (SE = 3.3 and 6.1%), respectively. HMMA recovery by acid hydrolysis was 336.1% and 159.8% greater than E. coli and H. pomatia β-glucuronidase (SE = 72.8 and 31.6%), respectively. The effects of temperature, time, and acid amount on metabolite recovery were also evaluated. HMA and HMMA acid hydrolysis recoveries were improved at 100°C and above. Effective hydrolysis could be conducted in a dry block heater, GC oven, or autoclave at temperatures from 100 to 140°C. Optimal hydrolysis conditions for the measurement of MDMA metabolite conjugates were addition of 100 μL of hydrochloric acid to 1 mL urine and incubation at 120°C in a GC oven for 40 min. Therefore, based on HMMA, HMA, and MDMA recoveries, time efficiency, availability of instrumentation, and cost, acid hydrolysis was preferred to enzyme hydrolysis.

Introduction

Primarily developed as an appetite suppressant in 1914, 3,4-methylenedioxyamphetamine (MDMA) is now an illegal narcotic banned in North America and Europe. MDMA has both stimulant and hallucinogenic properties (1,2) and is classified as an entactogen, a subgroup of drugs that enhance tactile responses (3). MDMA is excreted primarily as unchanged drug in human urine, and its metabolism and excretion in human urine have been previously described (4–8). N-Demethylation of MDMA produces 3,4-methylenedioxymethylamphetamine (MDA). Both MDMA and MDA are O-demethylated to 3,4-dihydroxymethylamphetamine (HHMA) and 3,4-dihydroxyamphetamine (HHA), respectively. HHMA and HHA are reported to be intermediate metabolites and are subsequently O-methylated to 4-hydroxy-3-methoxymethylamphetamine (HMMA) and 4-hydroxy-3-methoxymethamphetamine (HMA). HMMA is reported to be the major urinary metabolite (5,8) with HHMA contributing significant urinary concentrations approximately equal to unmetabolized MDMA. MDA and HHA are considered to be minor metabolites (8). HHMA, HHA, HMMA, and HMA are excreted as glucuronide and sulfate conjugates (5). HHMA and HHA are not commercially available and were not included in this study.

Measurement of MDMA metabolites is important for pharmacokinetic profiles and determining terminal elimination half-lives. MDMA metabolites must be cleaved from glucuronide/sulfate conjugates prior to extraction to maximize recovery. Two methods are commonly employed to cleave these conjugates; enzymatic hydrolysis with β-glucuronidase from Helix pomatia (H. pomatia) and hydrolysis with hydrochloric acid.

Although enzymatic (6,7) and acid (8) hydrolysis have been used to recover MDMA analogues in human urine, few studies have compared and reported optimal conditions for hydrolysis. Ortuno et al. (7) and Jenkins et al. (6) hydrolyzed MDMA metabolite conjugates with 20,000 units of H. pomatia type HP-2 β-glucuronidase per milliliter of urine incubated for 16 h at 37°C. Segura et al. (8) employed a 30 min acidic hydrolysis with 1 mL of 0.5M hydrochloric acid at 100°C. Helmlin et al. (9) compared autoclave acid hydrolysis (HCl 37%, 0.4 mL; 2 mL of urine), incubated for 15 min at 120°C and 105 Pa pressure to enzymatic hydrolysis (10,000 Fishman units of β-glucuronidase/sulfatase H. pomatia type H-1 per milliliter of urine incubated for 16 h at 37°C). Acid hydrolysis was found to be more efficient than H. pomatia enzymatic hydrolysis; however, quantitative recovery data were not reported (9).

The goal of this investigation was to optimize and compare enzymatic and acid hydrolysis of MDMA metabolites; HMA, MDA, and HMMA glucuronides; and sulfates in human urine. Experiments were conducted on urine specimens collected 2 to 48 h after oral MDMA administration. Specimens were...
hydrolyzed and assayed in duplicate under multiple enzymatic and acidic conditions.

Experimental

Materials

- β-Glucuronidase from H. pomatia (type HP-2) and Escherichia coli (E. coli) (type IX-A) were obtained from Sigma Chemical (St. Louis, MO). Solvents were high-performance liquid chromatography (HPLC) grade and obtained from Mallinckrodt Baker (Phillipsburg, NJ). Ammonium hydroxide, glacial acetic acid, hydrochloric acid, dibasic potassium phosphate, monobasic potassium phosphate, sodium acetate, and sodium hydroxide were ACS reagent grade and purchased from Mallinckrodt Baker. Solid-phase extraction (SPE) columns (Clean Screen ZSDAU020) and vacuum manifolds were obtained from United Chemical Technologies (Bristol, PA). Drug-free urine was obtained from volunteers and evaluated by gas chromatography–mass spectrometry (GC–MS) to ensure the absence of MDMA and metabolites.

Reference standards for MDMA, MDMA-d₅, MDA, MDA-d₅, and HMMA were obtained from Cerilliant (Austin, TX), and HMA was obtained from Lipomed (Cambridge, MA). All stock solutions were 1.0 g/L in methanol. Intermediate solutions of 100, 10, 1, and 0.1 μg/L were prepared in methanol. Heptafluorobutyric acid anhydride (HFBA) was purchased from Pierce Biotechnology (Parkville, IL).

Urines specimens

Clinical studies were conducted according to the World Medical Association’s “Ethical Principles for Medical Research involving Human Subjects”. The NIDA Intramural Research Program Institutional Review Board approved the protocol and participants provided informed consent and were compensated for participation. Urine specimens were collected prior to and after participation. Urine specimens containing internal standards were applied to preconditioned SPE columns. Columns were washed with deionized water, acetic acid, and methanol and dried under full vacuum for 5 min. Analytes were eluted with elution solvent (methylene chloride/isopropanol/concentrated ammonium hydroxide, 78:20:2, v/v). One percent hydrochloric acid in methanol was added to the eluate to reduce evaporative losses under nitrogen at 37°C. HFBA and ethyl acetate were added to the concentrated extracts for derivatization at 70°C for 10 min. One percent hydrochloric acid in acetonitrile was added, and extracts were evaporated. Derivatized analytes were reconstituted in heptane and injected onto the GC–MS.

GC–MS analysis

The analytical method for MDMA and metabolites is currently under consideration for publication (10). Urine specimens containing internal standards were prepared to simulate clinical samples. A calibration curve was prepared with 1 mL of certified negative urine with MDMA, HMA, MDA, and HMMA at 10, 25, 50, 100, 1000, 2500, 5000, and 10,000 μg/L.

No deuterated compounds were commercially available as internal standards for HMA and HMMA. MDA-d₅ and MDMA-d₅ were considered as internal standards for these analytes because of their common chemical structure. MDA-d₅ and HMA are primary amines, and MDMA-d₅ and HMMA are secondary amines. Both MDA-d₅ and MDMA-d₅ yielded reproducible results for HMA and HMMA. MDA-d₅ was selected as the internal standard for both analytes because of closer retention time (8.86 min) to HMA (8.04 min) and HMMA (9.20 min). MDMA-d₅ and MDA-d₅ (50 ng each) were added to each urine specimen prior to hydrolysis.

Enzymatic hydrolysis

Two milliliters of 134,300 units/mL stock H. pomatia β-glucuronidase was diluted to 25 mL with 0.1M potassium phosphate buffer (pH 5.0) to prepare working enzyme solution. H. pomatia working enzyme solution (940 μL) was added to each 1-mL urine sample (10,000 units/mL). Stock E. coli β-glucuronidase (500,000 units) was diluted to 50 mL with 0.1M potassium phosphate buffer (pH 6.8) to prepare E. coli enzyme solution. One milliliter of the enzyme solution was added to each 1-mL urine sample (10,000 units/mL). Tubes were capped, vortex mixed gently, and incubated for 16 h at 37°C in a shaking water bath. After allowing specimens to cool to room temperature, tubes were centrifuged at 1850 x g for 10 min.

Acid hydrolysis

One milliliter of each urine specimen was spiked with 100 or 200 μL of concentrated hydrochloric acid and incubated at 100, 110, 120, 140, or 160°C for 20, 30, 40, or 60 min. Tubes were incubated in a dry block heater, a GC oven, or in an autoclave at 10³ Pa pressure.

After allowing specimens to cool to room temperature, 250 μL of 10M sodium hydroxide and 3 mL of 0.1M phosphate buffer (pH 6.0) were added. Tubes were vortex mixed for 0.5 min and centrifuged at 1850 x g for 10 min.

Extraction, derivatization, and GC–MS analysis

The analytical method for MDMA and metabolites is currently under consideration for publication (10). Urine specimens containing internal standards were prepared and evaluated by gas chromatography–mass spectrometry (GC–MS) to ensure the absence of MDMA and metabolites.

No deuterated compounds were commercially available as internal standards for HMA and HMMA. MDA-d₅ and MDMA-d₅ were considered as internal standards for these analytes because of their common chemical structure. MDA-d₅ and HMA are primary amines, and MDMA-d₅ and HMMA are secondary amines. Both MDA-d₅ and MDMA-d₅ yielded reproducible results for HMA and HMMA. MDA-d₅ was selected as the internal standard for both analytes because of closer retention time (8.86 min) to HMA (8.04 min) and HMMA (9.20 min). MDMA-d₅ and MDA-d₅ (50 ng each) were added to each urine specimen prior to hydrolysis.

Optimization and comparisons of enzymatic and acid hydrolysis were investigated through the following studies.

Study 1. Enzymatic β-glucuronidase (H. pomatia or E. coli) versus acid hydrolysis. Recoveries of MDMA metabolites from urine specimens after hydrolysis with β-glucuronidase were compared to recoveries after acid hydrolysis. Enzymatic hydrolysis conditions were 10,000 units/mL H. pomatia or E. coli β-glucuronidase incubated for 16 h at 37°C in a shaking water bath. Acid hydrolysis was conducted with 200 μL con-
centrated hydrochloric acid per milliliter of urine for 20 min at 120°C in a dry block heater.

Study 2. Acid hydrolysis: high temperature dry block versus 120°C in an autoclave. Recoveries of MDMA metabolites from urine specimens after acid hydrolysis in a high temperature dry block were compared to recoveries in an autoclave. Acid hydrolysis conditions were 200 µL of hydrochloric acid incubated in a dry block for 20 min at 160°C or an autoclave (105 Pa) at 120°C for 15 min.

Study 3. Acid hydrolysis: various temperatures in dry block. Recoveries of MDMA metabolites from urine specimens after acid hydrolysis in a high temperature dry block were compared at 5 different temperatures. Acid hydrolysis conditions were 200 µL of hydrochloric acid incubated in a dry block for 20 min at 100, 110, 120, 140, and 160°C.

Study 4. Acid hydrolysis: high temperature GC oven versus autoclave at multiple incubation times. Recoveries of MDMA metabolites from urine specimens after acid hydrolysis in a GC oven were compared to recoveries in an autoclave for 30, 40, or 60 min in a GC oven were compared to recoveries in an autoclave for 15 and 30 min. Acid hydrolysis conditions were 200 µL of hydrochloric acid incubated for 30, 40, or 60 min at 120°C in GC oven and in an autoclave (105 Pa) for 15 or 30 min at 120°C.

Study 5. Acid hydrolysis: various incubation times in GC oven. Recoveries of MDMA metabolites from urine specimens after acid hydrolysis in a GC oven were compared at various incubation times. Acid hydrolysis conditions were 200 µL of hydrochloric acid incubated at 120°C in GC oven for 20, 40, and 60 min.

Study 6. Acid hydrolysis: volume of acid evaluation. Recoveries of MDMA metabolites from urine specimens after acid hydrolysis with 100 or 200 µL of hydrochloric acid in a GC oven at 120°C for 40 min were compared.

Study 7. Acid hydrolysis: interconversion study. Blank urine samples were individually spiked with 2.5, 5.0, or 10.0 µg of HMA, MDA, HMMA, or MDMA. Spiked samples were incubated with 100 µL of hydrochloric acid for 40 min at 120°C in GC oven, and analyzed to evaluate interconversion.

Statistical analysis

Results are presented as raw data in tables and figures, and in the text as mean ± SEM errors. For studies 1 to 5, mean quantitative data for all specimens in each hydrolysis condition were compared using one-way analysis of variance, followed by multiple comparison tests using Bonferroni correction. For study 6, the two groups of mean quantitative data were compared between each hydrolysis condition with the non-parametric Mann-Whitney test. All tests were performed using Prism version 3.02 (GraphPad Software, San Diego, CA) and were two-tailed, and P values of less than 0.05 were considered significant.

Results

Study 1

Recoveries of MDMA, MDA, HMA, and HMMA from urine specimens (n = 6) without hydrolysis and after acid and enzymatic hydrolysis are shown in Figure 1. There were no significant differences in MDMA concentrations between non-hydrolyzed urine specimens and acid and enzymatic hydrolyzed specimens (Figure 1A). There were no significant differences in MDA concentrations between non-hydrolyzed urine specimens and acid and H. pomatia enzymatically hydrolyzed specimens; however, a significant improvement in MDA recovery (P < 0.01) was noted with E. coli β-glucuronidase (Figure 1B).

HMA was recovered at concentrations greater than the method LOQ in only one of six non-hydrolyzed urine specimens. Compared to non-hydrolyzed specimens, HMA recovery was significantly higher (P < 0.01, P < 0.05, and P < 0.05, respectively) after acid, H. pomatia β-glucuronidase, and E. coli β-glucuronidase hydrolysis (996.2 to 1310.4% ± 337.2 to 376.0% higher) (Figure 1C). HMMA recovery was also significantly higher (P < 0.001 and P < 0.01, respectively) after acid and H. pomatia β-glucuronidase hydrolysis (476.7% ± 1128.0% and 2056.0% ± 688.5% higher, respectively) (Figure 1D) as compared to non-hydrolyzed specimens, although improvement in HMMA recovery after E. coli

![Figure 1](https://academic.oup.com/jat/article-abstract/30/8/563/714384/1)

Recoveries of MDMA (A), MDA (B), HMA (C), and HMMA (D) from human urine specimens (n = 6) collected 2 h (a), 5 h (b), 6 h (c), 7 h (d), 14 h (e), and 17 h (f) after controlled MDMA administration. Specimens were analyzed without hydrolysis and after acid and enzymatic hydrolysis. Acid hydrolysis was performed with 0.2 mL of hydrochloric acid per mL of urine at 120°C for 20 min. Enzymatic hydrolysis was conducted with 10,000 units/mL β-glucuronidase (source H. pomatia or E. coli) incubation at 37°C for 16 h.

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hydrolysis was not significant. HMMA recovery after acid hydrolysis was significantly increased as compared to *E. coli* or *H. pomatia* enzymatic hydrolysis (Figure 1D). The average increases in HMMA recovery after acid hydrolysis were 336.1% ± 72.8% and 159.8% ± 31.6% compared to *E. coli* or *H. pomatia* enzymatic hydrolysis, respectively.

**Study 2**
Comparison of recoveries of MDMA, MDA, HMA, and HMMA from urine specimens after acid hydrolysis in a 160°C dry block and in an autoclave is shown in Table I. There were no significant differences in MDMA concentrations between non-hydrolyzed urine specimens and acid hydrolysis in either dry block or autoclave. Overall mean quantitative data demonstrate no statistically significant difference between non-hydrolyzed and hydrolyzed MDA concentrations in the three urine specimens evaluated (Table I). However, specimen “b” (5 h) concentration increased substantially in MDA recovery after hydrolysis.

Compared to non-hydrolyzed conditions, there was a significant increase in HMA and HMMA concentrations after autoclave acidic hydrolysis (P < 0.05). HMA concentrations were below the LOQ without hydrolysis, and 241.6–399.5 μg/L after acid hydrolysis in the autoclave. HMMA recoveries were dramatically increased by an average of 3543.2% ± 1732.0% and 3723.7% ± 1239.8% after acid hydrolysis in a dry block and in an autoclave, respectively, compared to non-hydrolyzed specimens. There was no statistically significant difference in HMA and HMMA recoveries between dry block and autoclave acid hydrolysis.

Stability of the compounds under autoclave conditions was checked by spiking blank urine with HMA, MDA, HMMA, or MDMA at three different concentrations. There was no significant degradation or interconversion of the compounds during the autoclave process. All compounds quantified within ± 17% of target concentrations.

### Study 3
Recoveries of MDMA, MDA, HMA, and HMMA from urine specimens after acid hydrolysis at 100, 110, 120, 140, and 160°C in a dry block are shown in Figure 2. There were no significant differences in MDMA, MDA, and HMA concentrations after acid hydrolysis (200 μL of hydrochloric acid incubated in a dry heat block for 20 min) at 100, 110, 120, 140, and 160°C (Figure 2A–C). There was a significant increase in HMMA concentrations after 160°C, compared to 100, 110, 120, and 140°C conditions (P < 0.05) as illustrated in Figure 2D. HMMA concentrations after acid hydrolysis for 20 min at 160°C were 7105.6–15,083.4 μg/L ± 323.7–485.5 μg/L, and for example, 5875.7–11,154.0 μg/L ± 105.6–315.2 μg/L at 100°C. There were no statistically significant differences in HMMA recoveries at 100, 110, 120, or 140°C.

### Study 4
Recoveries of MDMA metabolites from urine specimens after acid hydrolysis incubated for 30, 40, or 60 min in a 120°C GC oven were compared to recoveries in an autoclave at 120°C for 15 and 30 min. There were no significant differences in MDMA concentrations between non-hydrolyzed urine specimens and acid hydrolysis in either dry block or autoclave. Overall mean quantitative data demonstrate no statistically significant difference between non-hydrolyzed and hydrolyzed MDMA concentrations in the five urine specimens evaluated (data not shown). However, two of the five urine specimens, including the same specimen “b” (5 h) previously reported in study 2, did demonstrate a substantial increase in MDA recovery after hydrolysis.

As expected, there were significant increases in HMA and HMMA concentrations after acid hydrolysis in a GC oven for 30, 40, and 60 min in an autoclave for 15 and 30 min (P < 0.05) compared to non-hydrolyzed specimens. HMA concentrations were significantly increased by 665.0% ± 36.4% and 732.0% ± 3.7%, and HMMA concentrations were significantly increased by 3543.2% ± 1732.0%, and 3723.7% ± 1239.8% after acid hydrolysis in a dry block and in an autoclave, respectively, compared to non-hydrolyzed specimens. There was no statistically significant difference in HMA and HMMA recoveries between dry block and autoclave acid hydrolysis.

### Table I. Comparison of Recoveries of MDMA*, MDA, HMA, and HMMA from Human Urine Specimens (n = 3) Without Hydrolysis and After Hydrolysis (0.2 mL hydrochloric acid per mL of urine) in a Dry Block at 160°C for 20 min and Acid Hydrolysis in an Autoclave (10^5 Pa) at 120°C for 15 min

<table>
<thead>
<tr>
<th></th>
<th>HMA (μg/L)</th>
<th>MDA (μg/L)</th>
<th>HMA (μg/L)</th>
<th>MDMA (μg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>b: 5 h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No hydrolysis</td>
<td>&lt; LOQ</td>
<td>334.5 ± 2.5</td>
<td>1101.1 ± 134.8</td>
<td>12913 ± 92.7</td>
</tr>
<tr>
<td>Acid hydrolysis at 160°C, dry heat block</td>
<td>182.9 ± 11.2</td>
<td>614.0 ± 6.0</td>
<td>16095.2 ± 443.5</td>
<td>26322.2 ± 169.6</td>
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<tr>
<td>Acid hydrolysis in autoclave</td>
<td>214.6 ± 3.5</td>
<td>639.2 ± 0.1</td>
<td>17952.8 ± 272.8</td>
<td>24015.5 ± 66.7</td>
</tr>
<tr>
<td>c: 7 h</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>No hydrolysis</td>
<td>&lt; LOQ</td>
<td>474.8 ± 5.0</td>
<td>185.3 ± 7.5</td>
<td>11794.8 ± 186.0</td>
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<tr>
<td>Acid hydrolysis at 160°C, dry heat block</td>
<td>192.6 ± 20.4</td>
<td>538.3 ± 56.8</td>
<td>10137.7 ± 804.9</td>
<td>10607.9 ± 69.2</td>
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<tr>
<td>Acid hydrolysis in autoclave</td>
<td>209.4 ± 18.7</td>
<td>628.5 ± 12.7</td>
<td>10597.0 ± 231.2</td>
<td>11120.7 ± 518.6</td>
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<tr>
<td>h: 33 h</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>No hydrolysis</td>
<td>&lt; LOQ</td>
<td>202.5 ± 1.3</td>
<td>1293 ± 3.0</td>
<td>14825 ± 8.9</td>
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<tr>
<td>Acid hydrolysis at 160°C, dry heat block</td>
<td>389.1 ± 5.2</td>
<td>199.2 ± 2.1</td>
<td>5164.0 ± 151.0</td>
<td>14288 ± 9.5</td>
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<tr>
<td>Acid hydrolysis in autoclave</td>
<td>399.5 ± 30.1</td>
<td>212.3 ± 5.1</td>
<td>5064.8 ± 106.1</td>
<td>14223 ± 38.1</td>
</tr>
</tbody>
</table>

* Abbreviations: MDMA, 3,4-methylenedioxymethamphetamine (Ecstasy); HMA, 4-hydroxy-3-methoxyamphetamine; MDMA, 3,4, methylenedioxymethamphetamine; and HMMA, 4-hydroxy-3-methoxymethamphetamine.

Urine specimens were collected 5 h (b), 7 h (d), and 33 h (h) after MDMA administration. Mean ± SEM.
increased 3177.5% ± 107.8% and 3204.2% ± 110.4% after acid hydrolysis in a GC oven and autoclave, respectively, compared to non-hydrolyzed specimens. However, there were no statistically significant differences in HMA and HMMA recoveries between acid hydrolysis performed either in a GC oven or an autoclave or for different incubation times.

Study 5
Recoveries of MDMA, MDA, HMA, and HMMA in urine specimens after acid hydrolysis in a GC oven at 20, 40, and 60 min are shown in Table II. There were no statistically significant differences in any analyte's concentration between acid hydrolysis (200 μL of hydrochloric acid incubated at 120°C) in a GC oven for 20, 40, or 60 min.

Study 6
Recoveries of MDMA, MDA, HMA, and HMMA from urine specimens after acid hydrolysis in a GC oven at 120°C for 40 min with 100 or 200 μL hydrochloric acid are shown in Table III. There were no significant differences in any analyte's concentration when acid hydrolysis was conducted with 1 mL of urine and either 100 or 200 μL of hydrochloric acid.

Study 7
Assays were performed to evaluate potential interconversion or degradation of MDMA and its metabolites, MDA, HMA, and HMMA. Urine specimens were incubated with 100 μL of hydrochloric acid for 40 min at 120°C in a GC oven. Blank urine samples were spiked with MDMA, MDA, HMA, or HMMA at concentrations of 2.5, 5.0, and 10.0 μg/L. No interconversion or artifact formation was evident for any MDMA analyte.

Discussion
MDMA and metabolites recovery experiments were conducted on human urine specimens collected 2 h (a), 6 h (c), and 17 h (f) after controlled MDMA administration. Specimens were analyzed after acid hydrolysis in a dry block heater at 100, 110, 120, 140, and 160°C. Acid hydrolysis performed with 0.2 mL of hydrochloric acid per mL of urine for 20 min.

<table>
<thead>
<tr>
<th>Specimen</th>
<th>HMA (μg/L)</th>
<th>MDA (μg/L)</th>
<th>HMMA (μg/L)</th>
<th>MDMA (μg/L)</th>
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<tr>
<td>e-14h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No hydrolysis</td>
<td>41.0 ± 1.2</td>
<td>2300.1 ± 28.2</td>
<td>157.9 ± 0.4</td>
<td>19840.5 ± 41.7</td>
</tr>
<tr>
<td>Acid Hydrolysis at 120°C, GC oven, 20 min</td>
<td>832.0 ± 33.9</td>
<td>2324.1 ± 26.2</td>
<td>17174.3 ± 38.0</td>
<td>20915.8 ± 356.5</td>
</tr>
<tr>
<td>Acid Hydrolysis at 120°C, GC oven, 40 min</td>
<td>943.0 ± 33.6</td>
<td>2465.8 ± 13.4</td>
<td>18757.1 ± 75.0</td>
<td>21626.3 ± 1300.5</td>
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<tr>
<td>Acid Hydrolysis at 120°C, GC oven, 60 min</td>
<td>995.6 ± 2.5</td>
<td>2450.6 ± 39.2</td>
<td>19154.5 ± 486.5</td>
<td>21019.2 ± 1047.3</td>
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<tr>
<td>h-33h</td>
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</tr>
<tr>
<td>No hydrolysis</td>
<td>47.4 ± 4.3</td>
<td>200.3 ± 2.9</td>
<td>129.3 ± 4.0</td>
<td>1197.1 ± 8.8</td>
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<td>Acid Hydrolysis at 120°C, GC oven, 20 min</td>
<td>348.6 ± 13.3</td>
<td>200.4 ± 0.9</td>
<td>3798.8 ± 135.5</td>
<td>1223.9 ± 25.8</td>
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<td>Acid Hydrolysis at 120°C, GC oven, 40 min</td>
<td>361.3 ± 1.7</td>
<td>204.4 ± 0.5</td>
<td>4124.7 ± 210.2</td>
<td>1214.2 ± 17.7</td>
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<tr>
<td>Acid Hydrolysis at 120°C, GC oven, 60 min</td>
<td>317.5 ± 60.8</td>
<td>203.9 ± 1.1</td>
<td>4184.4 ± 376.6</td>
<td>1191.3 ± 19.1</td>
</tr>
</tbody>
</table>

*Abbreviations: MDMA, 3,4-methylenedioxymethamphetamine (Ecstasy); HMA, 4-hydroxy-3-methoxyamphetamine; MDA, 3,4-methylenedioxymethamphetamine; and HMMA, 4-hydroxy-3-methoxyamphetamine.

Table II. Comparison of Recoveries of MDMA*, MDA, HMA, and HMMA from Human Urine Specimens (n = 2) after Acid Hydrolysis (0.2 mL hydrochloric acid per mL of urine) in a GC Oven at 120°C for 20, 40, and 60 min*

*Urine specimens were collected 14 h (e) and 33 h (h) after MDMA administration.

*Mean ± SEM.
higher concentrations of HMA and HMMA (Figure 1). HMA block and for 15 rain at 120~ at 10 s Pa in autoclave (Table I). The initial study compared hydrolysis with 0.2 mL hydrochloric acid per mL of urine for 20 min at 120°C in a dry block heater to enzymatic hydrolysis with H. pomatia and E. coli as detailed here. MDMA concentrations were not affected by hydrolysis because it is not excreted as a glucuronide or sulfate conjugate (7). When compared to concentrations of non-hydrolyzed specimens, each hydrolysis condition increased one or more metabolites; acid hydrolysis yielded higher concentrations of HMA and HMMA, E. coli increased HMA and MDA concentrations, while H. pomatia yielded higher concentrations of HMA and HMMA (Figure 1). HMA concentrations were increased by all three hydrolysis conditions within the same significant range. Although MDA concentrations were significantly increased only by E. coli hydrolysis (33.0% ± 8.4% higher compared to non-hydrolyzed), HMMA concentrations were significantly higher by acid hydrolysis (4767.7% ± 1128.0%) compared to non-hydrolyzed specimens. Therefore, based on recovery of HMMA and HMA, acid hydrolysis was selected as the preferred hydrolysis method. Furthermore, there were significant cost savings by hydrolyzing specimens with acid rather than the expensive β-glucuronidase enzymes.

Thus, acid hydrolysis was chosen for further optimization. Comparison of acid hydrolysis in a dry heat block versus autoclave was conducted by hydrolyzing urine specimens with 200 μL of hydrochloric acid for 20 min at 160°C in a dry block and for 15 min at 120°C at 10^5 Pa in autoclave (Table I). The stability of HMA, MDA, HMMA, and MDMA during autoclave conditions was also evaluated by spiking blank urine at three different concentrations. No degradation of compounds during the autoclave process was observed. Autoclave hydrolysis yielded higher but statistically not significant recoveries for HMA and HMMA, as compared to heating in the dry block (Table I). Unfortunately, with this method of hydrolysis, a few samples dried completely, potentially producing volatility losses. Furthermore, specimens were more difficult to handle during autoclaving and took longer to cool prior to extraction.

Next, we compared hydrolysis with 200 μL of hydrochloric acid, incubated for 20 min at 5 different temperatures; 100, 110, 120, 140, and 160°C (Figure 2). A temperature of 160°C significantly increased recovery of HMMA compared to other temperatures. There were no significant differences in HMMA recoveries at 100, 110, 120, or 140°C. Although HMMA recovery was improved at 160°C, this high temperature was excluded because a number of samples dried completely under these conditions, and there was a safety concern about potential glass breakage and “spitting” of liquid from the tubes during heating.

To avoid use of the higher 160°C temperature, we explored increasing incubation times. Acidic hydrolysis for 30, 40, or 60 min in a GC oven was compared to acid hydrolysis for 15 and 30 min in an autoclave. Compared to non-hydrolyzed specimen concentrations, results showed significantly higher amounts of HMA and HMMA after hydrolysis in a GC oven or autoclave at all incubation times. The use of an autoclave was less convenient than the GC oven because the extra hour after hydrolysis was required for specimens to reach room temperatures. Also, most analytical laboratories do not have available autoclave instrumentation. The GC oven allowed for consistent incubation conditions for the acidic hydrolysis of MDMA metabolite conjugates in human urine.

Table III. Comparison of Recoveries of MDMA*, MDA, HMA, and HMMA from Human Urine Specimens (n = 5) after Acid Hydrolysis with 0.1 mL or 0.2 mL Hydrochloric Acid per mL of Urine in a GC Oven at 120°C for 40 min

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Hydrochloric Acid (μL)</th>
<th>HMA (μg/L)</th>
<th>MDA (μg/L)</th>
<th>HMMA (μg/L)</th>
<th>MDMA (μg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-4</td>
<td>200 μL</td>
<td>281.0 ± 20.0</td>
<td>583.8 ± 6.5</td>
<td>18420 ± 446.4</td>
<td>7633.8 ± 14.7</td>
</tr>
<tr>
<td></td>
<td>100 μL</td>
<td>294.8 ± 17.7</td>
<td>702.1 ± 6.2</td>
<td>18682 ± 1845.4</td>
<td>7677.0 ± 535.4</td>
</tr>
<tr>
<td>4-14</td>
<td>200 μL</td>
<td>929.6 ± 98.0</td>
<td>2460.0 ± 41.0</td>
<td>16633 ± 1513.3</td>
<td>30973 ± 1430.7</td>
</tr>
<tr>
<td></td>
<td>100 μL</td>
<td>1036.1 ± 9.1</td>
<td>2414.3 ± 0.4</td>
<td>17527 ± 131.8</td>
<td>29835 ± 181.1</td>
</tr>
<tr>
<td>40-48</td>
<td>200 μL</td>
<td>416.7 ± 1.4</td>
<td>135.8 ± 1.6</td>
<td>4594.7 ± 22.8</td>
<td>551.7 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>100 μL</td>
<td>437.7 ± 10.9</td>
<td>132.3 ± 0.6</td>
<td>4182.0 ± 149.4</td>
<td>542.8 ± 9.0</td>
</tr>
<tr>
<td>4-48</td>
<td>200 μL</td>
<td>200.5 ± 10.6</td>
<td>141.7 ± 2.5</td>
<td>2126.0 ± 13.9</td>
<td>507.1 ± 13.8</td>
</tr>
<tr>
<td></td>
<td>100 μL</td>
<td>252.8 ± 15.9</td>
<td>137.3 ± 0.5</td>
<td>2050.9 ± 32.5</td>
<td>485.9 ± 3.7</td>
</tr>
<tr>
<td></td>
<td>40 μL</td>
<td>354.4 ± 11.8</td>
<td>272.0 ± 2.1</td>
<td>3479.5 ± 212.1</td>
<td>494.6 ± 6.5</td>
</tr>
<tr>
<td></td>
<td>10 μL</td>
<td>438.2 ± 0.4</td>
<td>263.0 ± 4.4</td>
<td>3723.8 ± 46.3</td>
<td>941.0 ± 20.4</td>
</tr>
</tbody>
</table>

* Abbreviations: MDMA, 3,4-methylenedioxymethamphetamine (Ecstasy); HMA, 4-hydroxy-3-methoxyamphetamine; MDA, 3,4-methylenedioxyamphetamine; and HMMA, 4-hydroxy-3-methoxymethamphetamine.

† Urine specimens were collected 4 h (i), 14 h (e), 40 h (j), 42 h (k), and 48 h (l) after MDMA administration.
acid. Finally, urine hydrolysis was compared with 100 or 200 μL of hydrochloric acid incubated for 40 min at 120°C in a GC oven. As shown in Table III, 100 and 200 μL of hydrochloric acid produced similar concentrations of HMA, MDA, and HMMA. One-hundred microliters of hydrochloric acid was selected as the optimum acid volume for hydrolysis.

Additional assays were performed to evaluate potential interconversions or degradation of analytes at the selected hydrolysis conditions. Blank urine specimens fortified with 0.05, 5.0 and 50.0 μg of MDMA, MDA, HMMA, or HMA were analyzed by adding 100 μL of hydrochloric acid for a 40-min incubation in a GC oven at 120°C. Analyte concentrations were within ± 20% of target indicating no significant change in analyte concentrations.

Among all acid hydrolysis tests (Figure 1 and Tables I and II), there were no significant differences in overall mean quantitative MDA concentrations between non-hydrolyzed urine specimens and acid hydrolyzed in either dry block, GC oven, or autoclave. However, two of the seven urine specimens evaluated had a substantial increase in MDA recovery after hydrolysis. We cannot readily explain this finding because concentrations of MDA glucuronide conjugates would be expected to increase over time. As we evaluate urine specimens collected following controlled MDMA administration, we will continue to address this interesting finding. Final hydrolysis conditions were selected on the basis of the best recoveries for all four analytes of interest and time efficiency, cost, and routine availability of equipment.

A limitation of the present study is that standard sources of MDMA metabolite conjugates are not commercially available. Thus, it is not possible for us to state that the final hydrolysis conditions achieved 100% recovery of analytes from glucuronide and sulfate conjugates. However, strengths of the current investigation are the analysis of authentic human urine specimens collected after controlled administration of MDMA and the choice of specimens collected at different times after drug administration. These specimens contain MDMA metabolites and glucuronide conjugates at various concentrations. Relative recoveries were determined for 12 clinical specimens from the same participant. Three different pools of clinical urine specimens collected after MDMA administrations also were used as in-house hydrolysis controls to monitor consistency and validity of the hydrolysis recovery process. Potential use of LC–MS could also be useful to identify the glucuronide metabolites.

This study determined optimum hydrolysis conditions for monitoring MDMA metabolite conjugates in urine. The hydrolysis procedure will be applied to specimens collected in an ongoing study of the pharmacodynamic effects and pharmacokinetics of oral MDMA. These data are relevant for the measurement of MDMA and metabolites in urine and for the characterization of urinary MDMA pharmacokinetics.

Conclusions

In conclusion, for the analysis of MDMA and metabolites in urine, MDA recoveries were significantly increased by E. coli β-glucuronidase (33.0% ± 8.4% higher compared to non-hydrolyzed), whereas HMA and HMMA recoveries significantly increased by 1310.4% ± 356.8% and 4767.7% ± 1128.0%, respectively, following acid hydrolysis. Optimal hydrolysis conditions for the measurement of MDMA metabolite conjugates were addition of 100 μL of hydrochloric acid to 1 mL of urine and incubation at 120°C in a GC oven for 40 min. Therefore, based on HMMA, HMA, and MDA recoveries, time efficiency, availability of instrumentation, and cost, acid hydrolysis was preferred to enzyme hydrolysis.

Acknowledgments

This research was supported by the Intramural Research Program, NIDA, NIH funds. We would like to acknowledge technical assistance of W. David Darwin and Deborah Price.

References